Genetics and Molecular Research

Genetic diversity analysis of *Aspergillus flavus* isolates from plants and air by ISSR markers

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ABSTRACT. *Aspergillus flavus* is one of the most abundant and widely distributed fungi on earth. *A. flavus* produces aflatoxins (AFs), which are toxic secondary metabolites. AFs have harmful effects on public health (humans and animals) and agricultural crops. Inter-simple sequence repeat (ISSR) markers were used to analyze the genetic diversity of 30 *A. flavus* isolates from five agricultural crops and air. Genetic similarity coefficients (GSC) ranged from 0.51 to 0.10 based on three ISSR markers for the isolates tested. *A. flavus* isolates grouped into 6, 5, and 3 clusters using the unweighted pair-group method with arithmetic average of three ISSR markers. This study suggests that ISSR biotechnology is a highly useful tool for characterizing genetic diversity of *A. flavus* isolated from different sources.

Key words: *Aspergillus flavus*; Inter-simple sequence repeats; Genetic diversity

INTRODUCTION

Aspergillus is one of the most economically important genera, involving more than 185 species with diverse impacts on the health of humans, animals, and plants. The majority of these species commonly inhabits foods, plants, and soil, and is even found in air (Yu et al., 2005). The delicate analysis of genetic diversity has considerable implications for molecular plant breeding strategies and biological control in plant disease programs (Kumar, 1999). Recently, advanced molecular biology techniques, such as DNA profiling based on amplified fragment length polymorphisms (AFLP) (Singh et al., 2015), restriction fragment length polymorphisms (RFLPs) (Mohankumar, et al., 2010), random amplification of polymorphic DNA (RAPD) (Mahmoud et al., 2014), microsatellites or simple sequence repeats (SSR) (Mushtaq et al., 2015), expressed sequence tag-simple sequence repeats (EST-SSR) (Wang et al., 2012), short tandem repeats (STR) (Houshyarfard et al., 2015), and inter-simple sequence repeats (ISSR) (Zhang et al., 2013; Chhotaray et al., 2015), have been widely used for the characterization of genetic diversity in *A. flavus*.

ISSR-PCR is a genotyping technique based on length variation in the regions between microsatellites, with high discriminatory power of amplified DNA (Prince, 2015). Primers can be various combinations of di-, tri-, tetra-, and penta-nucleotide repeats, targeting multiple genomic loci to amplify ISSR sequences of various sizes (Reddy et al., 2002). The method is rapid and highly reproducible (unlike RAPD) as well as being fast and relatively inexpensive and less time-consuming (unlike AFLP and RFLP) (Varga, 2006), with the complexity of SSR (Chen et al., 2008). It thus combines the advantages of RAPD, AFLP, and SSR (Abadio et al., 2012).

In plant-pathogenic fungi, ISSR technology has been used to characterize genetic diversity (Mahmoud et al., 2014), genetic structure (Rampersad, 2013; Gramaje et al., 2014), fingerprinting (identification) (Priyanka et al., 2014), population genetic structure (Hadrich et al., 2010), phylogenetic studies (Dutech et al., 2007), genome mapping (Chakravarty, 2011), and gene tagging (Ratnaparkhe et al., 1998).

In the present study, we aimed to 1) assess the characterization of the genetic diversity of *A. flavus* by using the ISSR-PCR technique, and 2) examine molecular relatedness among *A. flavus* isolates collected from seed borne fungi and air in Saudi Arabia and Egypt.

MATERIAL AND METHODS

Fungal isolates

Thirty isolates of *A. flavus* (Table 1) were collected from different sources. Twenty-five isolates were obtained from grains and seeds of agricultural crops, whereas five isolates were obtained from air. All isolates were from Saudi Arabia, except for five isolates from Egypt. *A. flavus* isolates were maintained and the production of mycelia was performed as described by Mahmoud et al (2014).

Genomic DNA extraction of *A. flavus* isolates

The *A. flavus* spore suspension (10^6 spores/mL) of every isolate was inoculated into double-layer (one solid and one liquid) media in 50-mm Petri dishes. The base solid medium was a film of PDA, and the top medium was liquid PYG. DNA was extracted according to the methods reported by Mahmoud (2015).

Table 1. Isolates of *Aspergillus flavus* used in this study.

No.	Accession	Origin	Source	Species name
1	Co1	Egypt	Cotton	*A. flavus*
2	Co2	Egypt	Cotton	*A. flavus*
3	Co3	Egypt	Cotton	*A. flavus*
4	Co4	Egypt	Cotton	*A. flavus*
5	Co5	Egypt	Cotton	*A. flavus*
6	S1	Saudi Arabia	Sorghum	*A. flavus*
7	S2	Saudi Arabia	Sorghum	*A. flavus*
8	S3	Saudi Arabia	Sorghum	*A. flavus*
9	S4	Saudi Arabia	Sorghum	*A. flavus*
10	S5	Saudi Arabia	Sorghum	*A. flavus*
11	S6	Saudi Arabia	Sorghum	*A. flavus*
12	A1	Saudi Arabia	Air	*A. flavus*
13	A2	Saudi Arabia	Air	*A. flavus*
14	A3	Saudi Arabia	Air	*A. flavus*
15	A4	Saudi Arabia	Air	*A. flavus*
16	A5	Saudi Arabia	Air	*A. flavus*
17	M1	Saudi Arabia	Maize	*A. flavus*
18	M2	Saudi Arabia	Maize	*A. flavus*
19	M3	Saudi Arabia	Maize	*A. flavus*
20	M4	Saudi Arabia	Maize	*A. flavus*
21	P1	Saudi Arabia	Peanut	*A. flavus*
22	P2	Saudi Arabia	Peanut	*A. flavus*
23	P3	Saudi Arabia	Peanut	*A. flavus*
24	P4	Saudi Arabia	Peanut	*A. flavus*
25	P5	Saudi Arabia	Peanut	*A. flavus*
26	Ca1	Saudi Arabia	Cashew	*A. flavus*
27	Ca2	Saudi Arabia	Cashew	*A. flavus*
28	Ca3	Saudi Arabia	Cashew	*A. flavus*
29	Ca4	Saudi Arabia	Cashew	*A. flavus*
30	Ca5	Saudi Arabia	Cashew	*A. flavus*

PCR amplification and sequencing of the internal transcribed spacer regions (ITS1-5.8S-ITS2) was conducted.

Fungal isolates were molecularly identified by their ITS regions (ITS1-5.8S-ITS2), and 5.8S rRNA sequences. The primers ITS-1 (5'-TCCGTAGGTGAACCTGCGTG-3') and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') were used for PCR. PCR conditions follow the methods of Gehlot et al. (2011). PCR amplification was conducted using a T Personal Thermocycler (Biometra, Göttingen, Germany). The amplified PCR products were sequenced using an automated ABI-Prism 377 DNA Sequencer (Applied Biosystems Inc., CA, USA). The sequences were aligned with MEGA 7.01 software and compared with the nucleotide sequences in GenBank for final identification. The phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987). Identification at the species level was based on ITS similarity as a percentage (Higgins et al., 2007).

ISSR PCR

PCR amplification of ISSRs was performed with the primers $(GTG)_5$, $(GACA)_4$, and $(AGAG)_4G$. The reaction amplifications were applied according to methods used by Mohamed et al. (2014). Sequences of primers are listed in Table 1. PCR products were loaded on a 1% agarose gel containing ethidium bromide in 1X TAE, then visualized with a gel documentation system (Bio-Rad Laboratories, Inc., CA, USA). The DNA gel was scanned using AAB software (AAB Software Ltd., CA, USA) and a 100-bp DNA ladder (New England BioLabs Inc., Hitchin, UK) was used as a marker. The similarity level was determined using the UPGMA method.

RESULTS

Identification of isolates

The two *A. flavus* isolates M3 and A5 (Figure 1) were identified and authenticated by molecular analysis of their ITS1, ITS2, and 5.8S rDNA regions. We inferred from the phylogenetic tree that *A. flavus* isolate M3 was classified into a clade that included *A. flavus* EU515148 and KF33940. The M3 isolate was closely related to *A. flavus* EU515148 with ITS sequence similarity of 99.9%. *A. flavus* isolate A5 was classified into a clade that included *A. flavus* KF562199 and KJ433668. The A5 isolate was closely related to *A. flavus* KJ433668, with ITS sequence similarity of 99.9%.

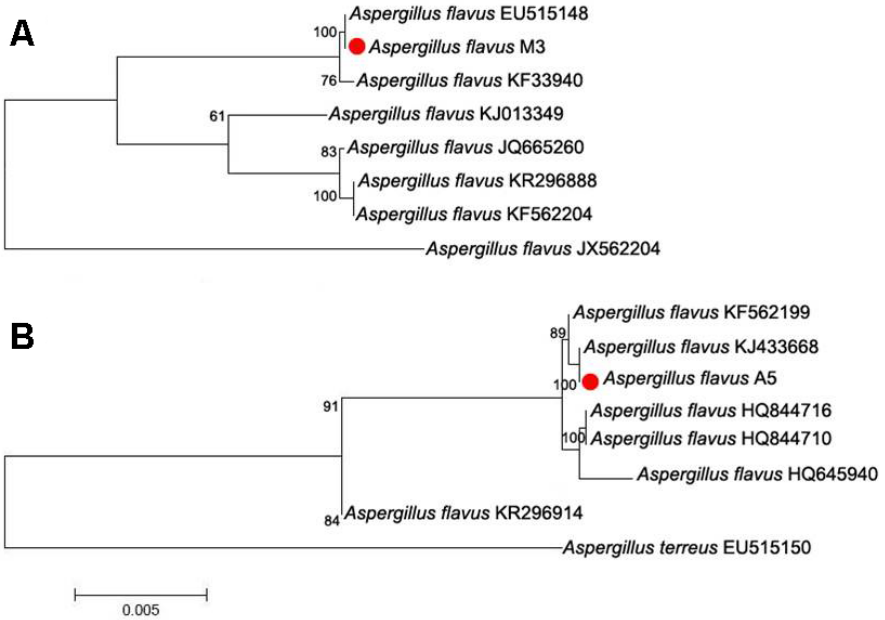

Figure 1. Phylogenetic tree of two *Aspergillus flavus* based on ITS region and 5.8S sequences. **A.** Isolate M3; **B.** isolate A5.

Genetic diversity of *A. flavus* using the $(GTG)_5$ primer

A cluster analysis was executed based on the GSC, which ranged from 0.41 to 1.0 for the 30 isolates (Figure 2). A dendrogram grouped 30 isolates into four main clusters (labeled A-D) using the UPGMA method. Four clusters were had GSC values of 0.49. Cluster A consisted of eight isolates, all from Saudi Arabia. Isolates A1 and A3 were from the air, and P1 and P3 were from peanut. The isolate Ca4 was from cashew, S6 from sorghum, and M3 from maize. Cluster B included 14 isolates, 12 isolates from Saudi Arabia and two from Egypt. These 12 isolates were from a variety of sources such as cashew, sorghum, and peanut. Cluster C included seven isolates, four isolates from Saudi Arabia and three from Egypt. Group C was divided into two subclusters. The first subcluster contained two isolates from cashew (Ca1 and Ca2) and one isolate from cotton (Co3). The second subcluster contained two isolates from cotton (Co2 and Co5) and two from sorghum (S1 and S4). Cluster D had one isolate M2 (maize) from Saudi Arabia.

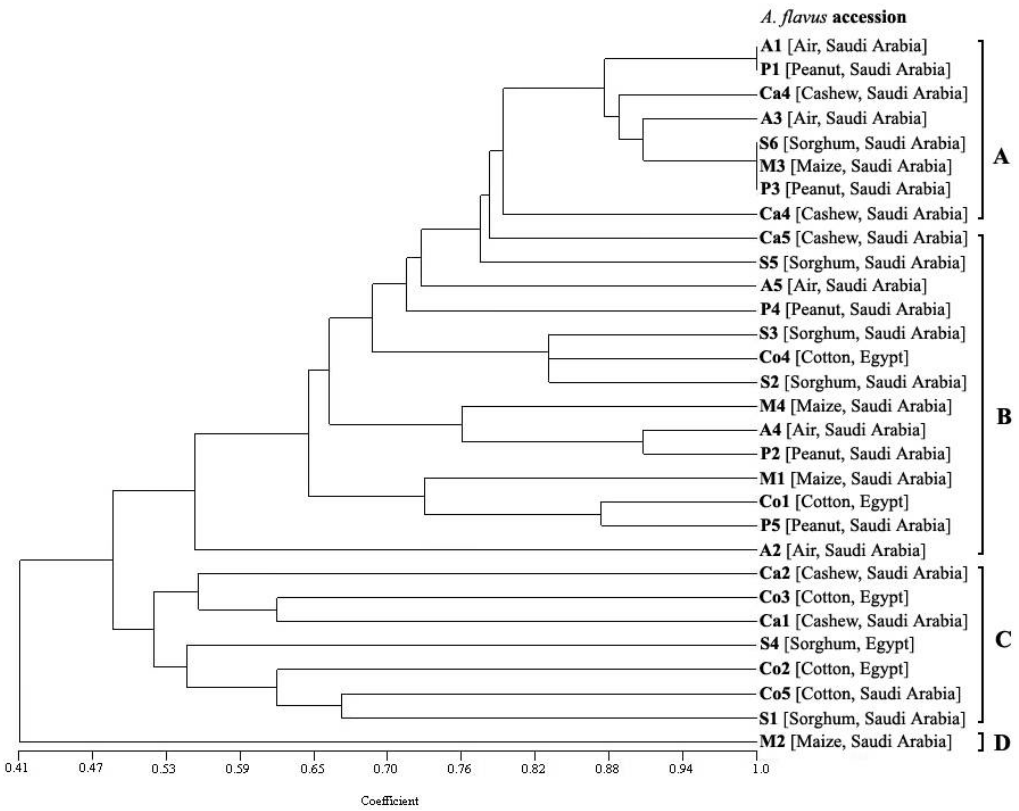

Figure 2. Dendrogram of 30 *A. flavus* isolates from UPGMA based on $(GTG)_5$ primer

Genetic diversity of *A. flavus* using the $(GACA)_4$ primer

The ISSR dendrogram was completed based on the GSC, which ranged from 0.29 to 1.0 for the 30 isolates (Figure 3).The ISSR dendrogram analysis grouped the 30 isolates into five main clusters (labeled A-F) using the UPGMA method. Five clusters were delineated at a GSC value of 0.38. Cluster A had the largest number of isolates (12 isolates), eleven from Saudi Arabia and one from Egypt. Cluster A was divided into two subclusters. The first subcluster contained nine isolates, and the second subcluster contained three isolates (M3, A4, and Co5). Cluster B included three isolates, all from Saudi Arabia. Cluster B consisted of A1, A2, and M1. Cluster C contained four isolates from Saudi Arabia: Ca4 and Ca5 (cashew), M2 (maize), and S5 (sorghum). Cluster D included nine isolates from different sources such as cotton, sorghum, and air. Clusters E and F contained one isolate each: P2 (peanut) and S4 (sorghum), both from Saudi Arabia.

Genetic diversity of *A. flavus* using the $(AGAG)_4G$ primer

The dendrogram generated using the GSC had values that ranged from 0.00 to 1.0 for the 30 isolates (Figure 4). The ISSR banding pattern showed three clusters (A-C). These three clusters were delineated at a GSC value of 0.38.

The genetic relationships among the 30 *A. flavus* isolates were also visualized by principal coordinate analysis of the three ISSR primers (Figure 5). The two-dimensional plot (Figure 5) generated from PCA supports the clustering pattern of the UPGMA dendrogram (Figure 5).

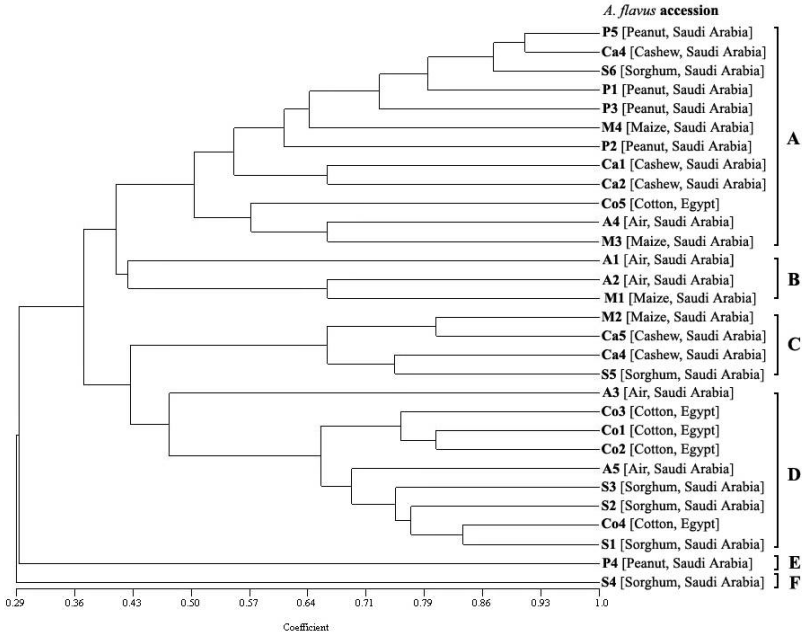

Figure 3. Dendrogram of 30 *A. flavus* isolates from UPGMA based on $(GACA)_4$ primer.

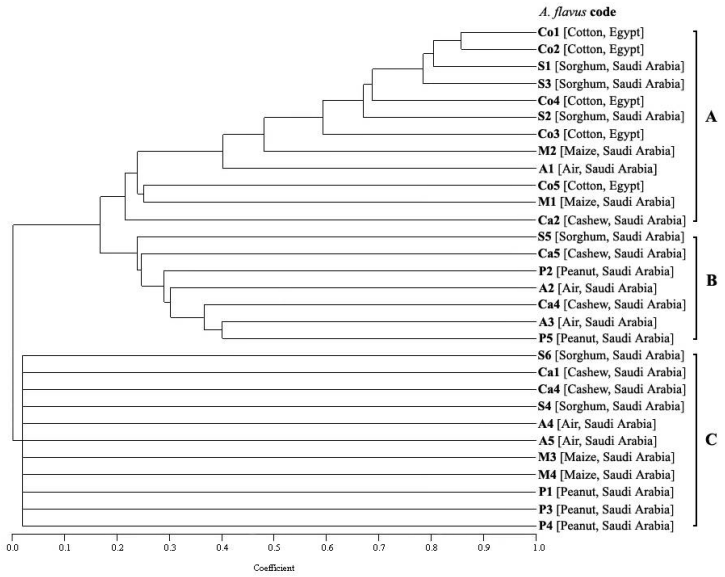

Figure 4. Dendrogram of 30 *A. flavus* isolates from UPGMA based on $(AGAG)_4G$ primer.

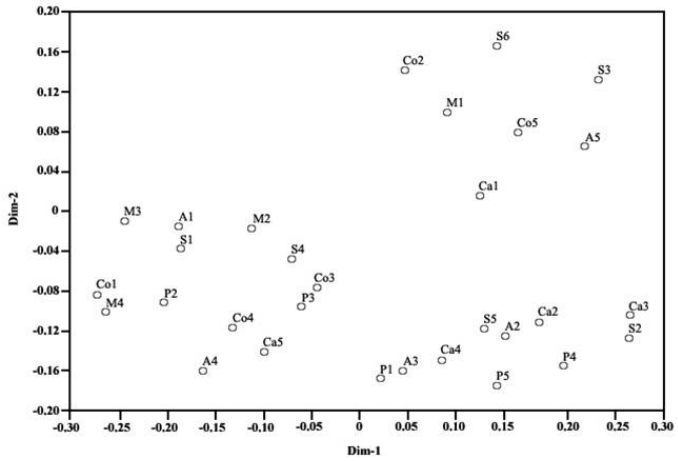

Figure 5. Principle coordinate analysis for 3 ISSR primers used to profile 30 *A. flavus* isolates.

DISCUSSION

A. flavus infects humans, animals, and plants (Yu et al., 2005). Aspergillosis is an umbrella term used to describe a wide range of diseases of human and animals (Schabereiter-Gurtner et al., 2007). *A. flavus* has a broad host range and acts as an opportunistic pathogen/saprobe for any crop seed (wheat, corn, sorghum, cotton, rice, and peanut) (Klich, 2007; Mahmoud, 2014).

Airborne *Aspergillus* species are the predominant myco-pollutants of outdoor air in Saudi Arabia (El-Samawaty et al., 2012). ISSR is a suitable molecular marking technique for the study of genetic diversity of plants (Reddy et al., 2002), bacteria (Rayar et al., 2015), and fungi (Mahmoud et al., 2014). Thirty-four *A. flavus* isolates were recovered from sorghum seeds sampled in India. These isolates were assessed using molecular markers, such as b-tubulin, ITS, and ISSR, to characterize molecular diversity. ISSR analysis showed a high level of molecular diversity without relation to geographic region or chemotype of aflatoxin production (Divakara et al., 2015).

Fifty-two isolates of *A. flavus* were collected from three crops (peanut, maize, and pistachio) and different geographical regions of Iran. ISSR technology was used to determine genetic relatedness and polymorphism between isolates. *A. flavus* isolates exhibited high intraspecific variability and a significant number of polymorphisms. The varying similarity ranges within isolates of *A. flavus* could also be a result of isolates that share a host range and/or ecological niche (Houshyarfard et al., 2015).

Three ISSR primers were used to evaluate the genetic variability of 15 *A. flavus* isolates from maize in Saudi Arabia. ISSR analysis revealed a high level of genetic diversity in the *A. flavus* population. The ISSR dendrograms obtained were unrelated to geographic origin or aflatoxin production (Mahmoud et al., 2014). Al-Wadai et al. (2013) obtained similar results from 13 *A. flavus* isolates from maize using the same three ISSR primers.

One hundred ISSR primers were evaluated for their ability to amplify DNA from 24 *A. flavus* isolates.

Twenty-two primers succeeded in generating polymorphic bands for each primer. The study showed that ISSR technology is an effective molecular approach for studying the diversity of *A. flavus* from soil of peanut crops in China (Zhang et al., 2013). A microsatellite analysis of

Vietnamese *A. flavus* strains (isolated from corn, peanut, and soil) revealed high genetic diversity. Clustering of microsatellite genotypes was unrelated to substrate and geographic origin or aflatoxin production (Tran-Dinh et al., 2009).

The primers $(GTG)_5$ and $(GACA)_4$ (ISSR primers) were used to study genetic diversity for 26 isolates of *Aspergillus* spp. ISSR markers detected high genetic diversity (inter- and intraspecific genetic variation), which is actually very useful as an auxiliary tool for genetic characterization of *A. flavus* strains (Batista et al., 2008).

Conflicts of interest

The authors declare no conflict of interest.

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